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Research Note—

Biologic Characterization of Chicken-Derived H6N2 Low Pathogenic Avian Influenza Viruses in Chickens and Ducks

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SUMMARY. Low pathogenic avian influenza H6N2 viruses were biologically characterized by infecting chickens and ducks in order to compare adaptation of these viruses in these species. We examined the clinical signs, virus shedding, and immune response to infection in 4-wk-old white leghorn chickens and in 2-wk-old Pekin ducks. Five H6N2 viruses isolated between 2000 and 2004 from chickens in California, and one H6N2 virus isolated from chickens in New York in 1998, were given intrachoanally at a dose of 1×10^6 50% embryo infectious dose per bird. Oral–pharyngeal and cloacal swabs were taken at 2, 4, and 7 days postinoculation (PI) and tested by real-time reverse-transcriptase polymerase chain reaction for presence of virus. Serum was collected at 7, 14, and 21 days PI and examined for avian influenza virus antibodies by commercial enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) testing. Virus shedding for all of the viruses was detected in the oral–pharyngeal swabs from chickens at 2 and 4 days PI, but only three of the five viruses were detected at 7 days PI. Only two viruses were detected in the cloacal swabs from the chickens. Virus shedding for four of the five viruses was detected in the oral–pharyngeal cavity of the ducks, and fecal shedding was detected for three of the viruses (including the virus not shed by the oral–pharyngeal route) in ducks at 4 and 7 days PI. All other fecal swabs from the ducks were negative. Fewer ducks shed virus compared to chickens. Both the chickens and the ducks developed antibodies, as evidenced by HI and ELISA titers. The data indicate that the H6N2 viruses can infect both chickens and ducks, but based on the number of birds shedding virus and on histopathology, the viruses appear to be more adapted to chickens. Virus shedding, which could go unnoticed in the absence of clinical signs in commercial chickens, can lead to transmission of the virus among poultry. However, the viruses isolated in 2004 did not appear to replicate or cause more disease than earlier virus isolates.

RESUMEN. *Nota de Investigación*—Caracterización biológica en pollos y patos de virus de la influenza aviar de baja patogenicidad H6N2 derivados de pollos.

Se caracterizaron de manera biológica los virus de la influenza aviar de baja patogenicidad H6N2 mediante la infección de pollos y patos para comparar la adaptación de estos virus en estas especies. Se examinaron los signos clínicos, la eliminación viral y la respuesta inmune a la infección en aves leghorn de cuatro semanas de edad y en patos Pekín de dos semanas de edad. Cinco virus H6N2 que fueron aislados entre los años 2000 y 2004 en pollos de California y un virus H6N2 aislado de pollos en Nueva York en el año 1998, fueron administrados por vía intracoanal en una dosis de 1×10^6 dosis infectantes 50% por ave. Se recolectaron hisopos orofaríngeos y cloacales a los 2, 4 y 7 días postinoculación y se analizaron por la prueba de transcripción reversa y reacción en cadena de la polimerasa en tiempo real para detectar la presencia del virus. Se recolectaron muestras de sueros a los días 7, 14 y 21 después de la inoculación y se examinaron para detectar anticuerpos mediante la prueba de inmunoabsorción con enzimas ligadas (ELISA) y por la prueba de inhibición de la hemaglutinación (HI). Se detectó eliminación viral con todos los virus en los hisopos orofaríngeos a los días 2 y 4 después de la inoculación, pero solo tres de los cinco virus se detectaron a los 7 días post inoculación. Solo dos virus se detectaron en los hisopos cloacales de los pollos. Se detectó eliminación viral en la cavidad orofaríngea de los patos con cuatro de los cinco virus y la eliminación fecal se detectó con tres virus (incluyendo los virus no eliminados por la ruta orofaríngea) en los patos a los 4 y 7 días postinoculación. Todos los otros hisopos fecales de los patos fueron negativos. Un número menor de patos eliminaron virus en comparación con los pollos. Los pollos y los patos desarrollaron anticuerpos, tal como se hizo evidente por los títulos de HI y ELISA. Estos datos indican que los virus H6N2 pueden infectar pollos y patos, pero de acuerdo con el número de aves que eliminaban virus y a la histopatología, estos virus parecen estar más adaptados a los pollos. La eliminación viral, que puede pasar desapercibida por la ausencia de signos clínicos en pollos comerciales puede facilitar la transmisión viral en la avicultura comercial. Sin embargo, los virus aislados en el año 2004 no parecen poseer una replicación mayor o causar enfermedad más severa que los virus aislados previamente.

Key words: avian influenza virus, H6N2, chickens, ducks, California live bird market, biologic characterization, pathogenicity

Abbreviations: AIV = avian influenza virus; Ct = cycle threshold; EID_{50} = 50% embryo infectious dose; ELISA = enzyme-linked immunosorbant assay; HI = hemagglutination inhibition; LPAI = low pathogenicity avian influenza viruses; PBS = phosphate-buffered saline; PI = postinoculation; RT-PCR = reverse transcriptase-polymerase chain reaction; SPF = specific-pathogen-free

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Avian influenza viruses (AIV) are worldwide in distribution and continue to be a threat to commercial poultry as well as to human health. Wild aquatic birds (Charadriiformes and Anseriformes), which include shorebirds, ducks, geese, and swans, are the natural reservoirs of AIV and play an important role in the ecology of the virus. Sometimes, these AIVs transmit from wild aquatic to domestic birds, producing subclinical infections and, occasionally, respiratory disease and drops in egg production. These viruses are typically classified as low pathogenicity avian influenza viruses (LPAI) in standard intravenous pathogenicity studies in chickens. The viruses in wild birds include at least 16 antigenically distinct hemagglutinin subtypes, and most have been isolated from poultry. Only a few H5 and H7 LPAI viruses have mutated to produce highly pathogenic avian influenza viruses after circulating in domestic poultry (11). Although little or no disease is usually associated with LPAI viruses in commercial chickens, they can infect, and are shed by, the birds (10). Some LPAI viruses have adapted to efficient replication in poultry, causing more prominent clinical signs and mortality (2).

The H6 subtype viruses are present in ducks and presumably can transmit to commercial chickens (4,8,13). In February 2000, an outbreak of H6N2 LPAI occurred in California, and 12 separate incidences were reported which primarily involved layer-type birds, but viruses were also isolated from backyard chickens and a primary broiler breeder (3,15). A drop in egg production and an increase in mortality were among the clinical signs reported in the layer flocks; however, the pathologic changes observed in theses earlier cases were primarily associated with mild respiratory infections. Yolk peritonitis was a feature later described for cases in 2001 and 2002 (15). The H6N2 subtype LPAI viruses continued to be detected in commercial chickens in California in 2002, 2003, and 2004, and an autogenous killed vaccine was developed to eradicate the virus (4).

The LPAI California H6N2 viruses, isolated from 2000 to 2002, likely had a common ancestor based on the close sequence similarity of the hemagglutinin gene, but analysis of all eight gene segments demonstrated different constellations of genes, likely from reassortment viruses between aquatic bird and chicken AIVs (14). In addition, those LPAI H6N2 viruses circulating between 2000 and 2002 were shown to have an 18 amino acid deletion in the neuraminidase protein, which is associated with adaptation to growth in chickens; however, the isolates were reported to be nonpathogenic in chickens by standard intravenous pathotyping studies (1,13,14).

In this study, we were interested in biologically characterizing H6N2 LPAI viruses isolated from chickens in California in 2000, 2002, and 2004 in order to determine adaptation of these viruses for chickens by examining infection and replication, and also to see if, by adaptation to chickens, these viruses are less adapted to ducks. One H6N2 LPAI virus isolate from New York was also studied for comparison purposes. We examined the clinical signs, virus shedding, adaptive immune response, and microscopic lesions in 4-wk-old white leghorn chickens and in 2-wk-old Pekin ducks challenged with the viruses.

MATERIALS AND METHODS

Viruses. All of the viruses used in this study were LPAI viruses. The virus designations and titers are presented in Table 1. Isolate A/CK/NY/14677-13/98 was the only virus examined from outside of California (6). Isolates from California include A/CK/CA/431/00 (15), isolated from a rooster in a back yard flock (thought to be the initial case); A/CK/CA/139/01 (14,15), isolated from a chicken in a mixed flock that included ducks and squabs; A/CK/CA/1255/02 (15), isolated from 118 1-wk-old layers; A/CK/CA/6028/04 from a bird at a live-bird-market custom slaughter plant; and A/CK/CA/7211/04 from a bird in a feed and pet supply store in Los Angeles that was in close proximity to the custom slaughter plant where A/CK/CA/6028/04 was isolated (Woolcock, pers. comm.).

Table 1. Viruses examined in this study.

Virus isolate	GenBank accession no.	Titer (EID_{50}/ml)
CK/NY/14677-13/98[A]	DQ021663	$1 \times 10^{5.9}$
CK/CA/431/00	AF474039	$1 \times 10^{8.0}$
CK/CA/139/01	AF457711	$1 \times 10^{8.0}$
CK/CA/1255/02[B]	GQ358535	$1 \times 10^{5.5}$
CK/CA/6028/04	GQ358533	$1 \times 10^{8.3}$
CK/CA/7211/04	GQ358535	$1 \times 10^{7.9}$

[A]Only ducks were exposed to this isolate.
[B]Only chickens were exposed to this isolate.

Experimental design. A minimum of eight, 4-wk-old SPF white leghorn chickens (Merial Select, Gainesville, GA) and 2-wk-old Pekin ducks (Metzer Farms, Gonzalas, CA) per group were used in this study. All experiments were conducted in a biosafety level 2 Ag+ facility at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, the University of Georgia (Athens, GA) in accordance with U.S. Department of Agriculture's Animal and Plant Health Inspection Service permit number 103372. Birds were housed in stainless steel and polycarbonate, negative pressure, HEPA filtered, isolator units with internal dimensions of 42'' L × 24'' W × 31'' H.

Viruses were diluted with sterile phosphate-buffered saline (PBS, pH 7.4) to adjust the amount of inoculum to 1×10^6 50% embryo infectious dose (EID_{50}) per 0.1 ml per bird. For viruses below 1×10^6 EID_{50} per 0.1 ml concentration, 0.1 ml of undiluted virus per bird was given. The viruses were administered via the oropharyngeal (intrachoanal) route. For each experiment, one group of birds was not inoculated and served as negative controls. After inoculation, all birds were observed for clinical signs of disease and mortality twice daily for 21 days. Clinical signs of disease were scored and recorded as follows: 0 = no signs; 1 = mild to moderate respiratory signs; 2 = moderate respiratory signs, depressed or not eating; 3 = moderate to severe respiratory signs, depressed, not eating, and neurologic signs; 4 = moribund birds (were removed and necropsied immediately).

Oropharyngeal and cloacal swabs were collected in 1 ml of sterile PBS from each bird at 2, 4, and 7 days postinoculation (PI), stored at −80 C, and thawed only once for RNA extraction. Serum was also collected from each bird at 7, 14, and 21 days PI and stored at −20 C. Tissue samples for histopathology were collected from three birds per group at 3 days PI.

RNA extraction and quantitative, real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Viral RNA was extracted from swab samples using the MagMax-96 Total RNA isolation kit (Ambion Inc., Austin, TX) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturer's recommendations.

The Ambion Ag Path ID One Step RT-PCR kit (Ambion Inc.) was used for nucleic acid amplification with a 25-μl reaction mixture containing the following reagents: 12.5 μl of kit-supplied 2× RT-PCR buffer, 1 μl of kit-supplied 25× RT-PCR enzyme mix, and 10 μl of extracted viral RNA. Each reaction mixture utilized 10 picomoles of matrix gene primers (forward and reverse) and probe sequences (all three using 0.5 μl each), following the protocol of the real time RT-PCR assay developed for type A influenza virus (5). Real-time RT-PCR was carried out in a Smart Cycler thermocycler machine (Cepheid, Sunnyvale, CA) with the following conditions for the RT step (50 C for 30 min and 94 C for 15 min) and the PCR cycling protocol (94 C for 15 sec and 60 C for 20 sec for 45 cycles). Data were reported as the average cycle threshold (Ct) value.

Histopathology. Microscopic examination was conducted on the following tissues: heart, lung, liver, spleen, pancreas, duodenum, jejunum, cecum, cecal tonsils, ileum, bursa of Fabricius, breast and thigh muscle, thymus, nasal cavity, and brain. Tissue samples were fixed

Table 2. Virus detection in chickens presented as real-time RT-PCR cycle threshold values.[A]

	Days postchallenge					
	Day 2		Day 4		Day 7	
Virus isolate	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
A/CK/CA/431/00	31.6/5.7 (8/10)[B]	Neg (0/10)	28.7/6.6 (7/7)	Neg (0/7)	32.3/5.6 (2/7)	31.2/5.9 (1/7)
A/CK/CA/139/01	28.4/6.68 (10/10)	37.3/4.2 (1/10)	29.5/6.4 (7/7)	34.4/5.0 (1/7)	Neg (0/7)	Neg (0/7)
A/CK/CA/1255/02	30.25/6.2 (10/10)	Neg (0/10)	33.8/5.2 (7/7)	Neg (0/7)	35.8/4.6 (1/7)	Neg (0/7)
A/CK/CA/6028/04	32.1/5.6 (10/10)	Neg (0/10)	33.2/5.3 (7/7)	Neg (0/7)	33.7/5.2 (2/7)	Neg (0/7)
A/CK/CA/7211/04	33.1/5.4 (9/10)	Neg (0/10)	34.1/5.1 (7/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)
Controls	Neg (0/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)

[A]Average Ct value for the positive birds (values greater than 38.5 were considered negative [Neg])/relative viral equivalents ($\log_{10}$)/ml calculated from the average Ct value and a standard curve for the H6 LPAI viruses ($y = -0.278x + 14.58$).
[B]Number positive per total examined.

in 10% neutral-buffered formalin, routinely processed, and embedded into paraffin blocks. Thin sections were cut and stained with hematoxylin and eosin and examined by light microscopy.

Serologic testing. Chicken sera were tested for antibodies to AIV using a commercial enzyme-linked immunosorbant assay (ELISA) kit, the FlockChek™ avian influenza virus antibody test (IDEXX, Portland, ME). Duck sera were tested using the avian influenza MultiS-Screen ELISA kit (IDEXX). In addition, samples were tested for antibodies by the hemagglutination inhibition (HI) test using 4 HA units of the CK/CA/431/00 virus (11).

Sequencing. The HA1 region of the influenza HA genes were amplified by RT-PCR as previously described (9). The RT-PCR products were purified by agarose gel extraction with the Qiaquick gel extraction kit (Qiagen, Inc., Valencia, CA) and were directly sequenced. The BigDye terminator kit (Applied Biosystems, Foster City, CA) was used for cycle sequencing and subsequently run on an ABI 3730 sequencer (Applied Biosystems).

Phylogenetic and sequence analysis. The HA1 coding region of the HA genes (nucleotides: 1–1035) were aligned with Clustal V (Lasergene, V. 8.0.2 DNAStar, Inc., Madison WI). The phylogenetic tree was generated using the neighbor-joining distance method (DNAStar, Inc.) and confirmed by the maximum parsimony method with 1000 bootstrap replicates in a heuristic search using the PAUP 3.1 software program (MacDNASIS, Hatachi Software Engineering America, Ltd., San Bruno, CA).

RESULTS AND DISCUSSION

The titers of the viruses used to challenge the chickens and ducks are presented in Table 1. For comparison purposes, we wanted to use the same titered virus stock for both chickens and ducks. Unfortunately, two of the viruses, CK/NY/14677-13/98 and CK/CA/1255/02, grew to low titers in eggs and, thus, were only given to ducks and chickens, respectively, because there was not enough virus from the same stock to infect both species. All of the other viruses were given to both chickens and ducks. No clinical signs were observed in either species during the experiment, which is typical of LPAI viruses (7,10). Two of the viruses (CK/CA/431/00 and CK/CA/139/01) were previously shown to be infectious in experimentally exposed chickens, but did not cause any overt clinical signs (14).

The virus detection data is presented in Table 2 (chickens) and Table 3 (ducks). No virus was detected in the nonchallenged control birds. All of the viruses given to the chickens were detected in the oropharyngeal swabs. With the exception of two birds given CK/CA/431/00 and one bird given CK/CA/7211/04, both necropsied on day 2 PI, all of the oropharyngeal swabs from the chickens were positive on days 2 and 4 PI. Virus was not detected in the oropharyngeal swabs from most of the chickens by day 7 PI. Previously, the CK/CA/139/01 virus was reported to have been recovered from the cloacae of an infected chicken after an intravenous challenge (14). We found CK/CA/139/01 in the cloacal swab from one chicken on day 2 PI and from one chicken on day 4 PI. We also detected virus in the cloacal swab from one chicken given CK/CA/431/00 at day 7 PI. Virus was not detected in the cloacal swabs from any of the other chickens.

In the ducks, virus was detected in the oropharyngeal swabs for four of the five viruses, and three viruses, CK/NY/14677-13/98, CK/CA/139/01, and CK/CA/6028/04, induced shedding from the cloaca, which was detected at day 4 postchallenge for the CK/NY/14677-13/98 virus and at day 7 postchallenge for the CK/CA/139/01 and CK/CA/6028/04 viruses. In general, the Ct values for virus samples from chickens were lower (indicating relatively more virus) than the samples from the ducks, and a greater number of chickens were infected, compared to ducks, for the viruses that were given to both species. Taken together, these data indicate that the viruses

Table 3. Virus detection in ducks presented as real-time RT-PCR cycle threshold values.[A]

	Days postchallenge					
	Day 2		Day 4		Day 7	
Virus isolate	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
A/CK/NY/14677-13/98	27.4/7.0 (3/10)[B]	Neg (0/10)	30.2/6.2 (7/7)	19.0/9.29 (7/7)	34.5/5.0 (4/7)	Neg (0/7)
A/CK/CA/431/00	29.0/6.5 (3/10)	Neg (0/10)	32.6/5.5 (1/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)
A/CK/CA/139/01	33.3/5.3 (2/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	32.5/5.5 (1/7)
A/CK/CA/6028/04	Neg (0/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	27.9/6.8 (5/7)
A/CK/CA/7211/04	33.4/5.3 (4/10)	Neg (0/10)	29.3/6.4 (1/7)	Neg (0/7)	37.4/4.2 (1/7)	Neg (0/7)
Controls	Neg (0/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)

[A]Average Ct value for the positive birds (values greater than 38.5 were considered negative [Neg])/relative viral equivalents ($\log_{10}$)/ml calculated from the average Ct value and a standard curve for the H6 LPAI viruses ($y = -0.278x + 14.58$).
[B]Number positive per total examined.

Table 4. Hemagglutination inhibition (HI) antibody titers against avian influenza virus and the number of serologically positive chickens by ELISA.

	Days postchallenge					
	Day 7		Day 14		Day 21	
Virus isolate	HI[A]	ELISA[B]	HI	ELISA	HI	ELISA
A/CK/CA/431/00	64.6 (5/7)	1/7	476.9 (7/7)	5/7	749.7 (7/7)	2/7
A/CK/CA/139/01	71.6 (7/7)	3/7	50.6 (7/7)	2/7	72.3 (7/7)	4/7
A/CK/CA/1255/02	40.3 (7/7)	2/7	41.1 (7/7)	5/7	67.8 (7/7)	5/7
A/CK/CA/6028/04	126.8 (7/7)	4/7	87.1 (6/7)	1/7	78.0 (6/7)	5/7
A/CK/CA/7211/04	40.7 (6/7)	2/7	82.3 (7/7)	4/7	121.1 (7/7)	4/7
Controls	Neg (0/7)	0/7	Neg (0/7)	0/7	Neg (0/7)	0/7

[A]Mean HI titer of the positive samples (number positive/total). HI testing was done with 4 HA units of the CK/CA/431/00 virus and positives were considered to be ≥8. Neg = negative.

[B]Number of positive birds/total examined. Positive samples were determined from the sample-to-positive ratio, as calculated by the manufacturer's software (IDEXX Laboratories).

Table 5. Hemagglutination inhibition (HI) antibody titers against avian influenza virus and the number of serologically positive ducks by ELISA.

	Serology					
	Day 7		Day 14		Day 21	
Virus isolate	HI[A]	ELISA[B]	HI	ELISA	HI	ELISA
A/CK/NY/14677-13/98	45.7 (3/7)	6/7	31.6 (7/7)	7/7	31.6 (5/7)	7/7
A/CK/CA/431/00	54.8 (7/7)	5/7	70.8 (7/7)	4/7	69.3 (5/7)	5/7
A/CK/CA/139/01	80.0 (6/7)	5/7	31.6 (6/7)	3/7	27.4 (6/7)	4/7
A/CK/CA/6028/04	16.4 (2/7)	3/7	20.0 (2/7)	0/7	12.0 (2/7)	0/7
A/CK/CA/7211/04	71.6 (6/7)	7/7	19.3 (4/7)	2/7	48.2 (6/7)	2/7
Controls	Neg (0/7)	0/7	Neg (0/7)	0/7	Neg (0/7)	0/7

[A]Mean HI titer of the positive samples (number positive/total). HI testing was done with 4 HA units of the CK/CA/431/00 virus and positives were considered to be ≥8. Neg = negative.

[B]Number of positive birds/total examined. Positive samples were determined from the sample-to-positive ratio, as calculated by the manufacturer's software (IDEXX Laboratories).

were infectious and shed at higher levels in chickens than in ducks. In addition, it appears that virus replication and shedding from the oropharynx, rather than from the cloaca, is a characteristic of theses viruses in chickens and ducks.

Although the true LPAI virus dose that can sustain transmissibility is unknown, the mean intranasal bird infectious dose for selected LPAI viruses was previously reported to range from 3.0 to 7.7 in chickens and from 1.9 to 3.3 in ducks, and the dose varied with the virus isolates (12). Calculated relative viral equivalents, based on Ct values, indicated that an infectious dose was being shed by some of the birds in our experiments (Tables 2, 3).

Serology results are presented in Tables 4 and 5, for the chickens and ducks, respectively. None of the nonchallenged birds had detectable antibody titers against AIV. Although the HI titers for the viruses given to both species were, on average, higher in chickens than in ducks, more ducks seroconverted based on ELISA testing. It appears that the ducks were more susceptible to infection with these viruses, but virus replication in the ducks was lower than in the

Table 6. Severity of microscopic lesions found in tissues of chickens infected with H6N2 LPAI viruses.[A]

	Virus				
Tissue	A/CK/CA/431/00	A/CK/CA/139/01	A/CK/CA/1255/02	A/CK/CA/6028/04	A/CK/CA/7211/04
Nasal cavity	++[B]	++	+	++	++
Trachea	+	++	++	+	+
Lung	+	+	+	+	++
Heart	+/−	−	−	+/−	+
Brain	−	−	−	−	−
Enteric tract	+	+	+	++	+
Pancreas	+	+	+/−	+	+/−
Liver	++	++	+	+	+
Kidney	−	−	−	−	−
Spleen	−	−	−	−	−
Bursa	+/−	+/−	+/−	+	+/−
Thymus	+/−	+	−	+/−	+/−
Muscle	−	−	−	−	−
Gonads	+	+	+/−	+/−	+

[A]Tissues collected from three birds at day 3 PI.

[B]Average severity of lesions; − = no lesions; +/− = minimal; + = mild; ++ = moderate; +++ = severe lesions.

Table 7. Severity of microscopic lesions found in tissues of ducks infected with H6N2 LPAI viruses.[A]

Tissue	Virus					
	A/CK/NY/14677/89	A/CK/CA/431/00	A/CK/CA/139/01	A/CK/CA/1255/02	A/CK/CA/6028/04	A/CK/CA/7211/04
Nasal cavity	+[B]	+	+	+	+	++
Trachea	++	++	++	++	+	++
Lung	+/−	+/−	−	−	+	+/−

[A]Tissues collected from three birds at day 3 PI.
[B]Average severity of lesions; − = no lesions; +/− = minimal; + = mild; ++ = moderate; +++ = severe lesions.

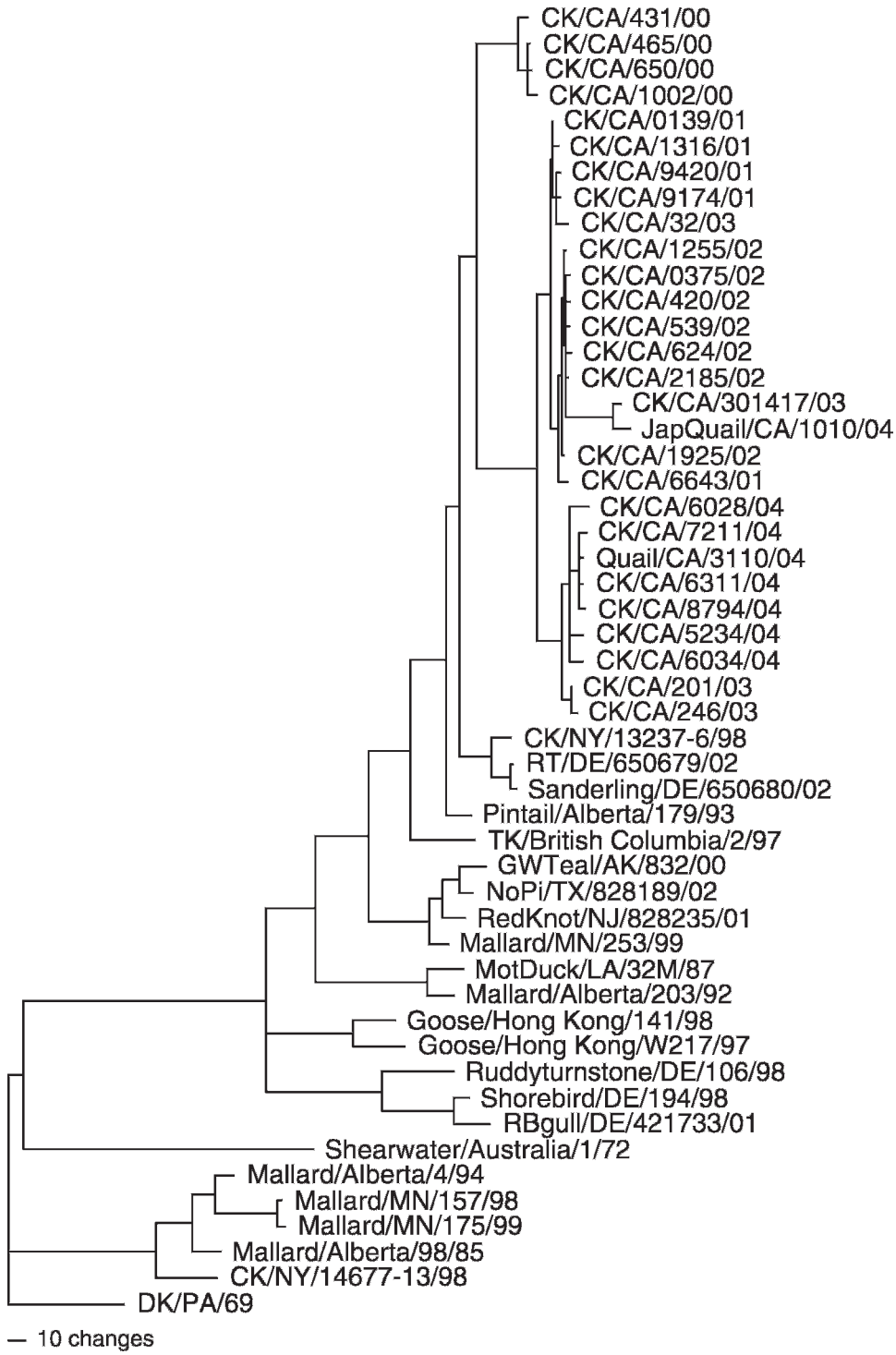

Fig. 1. Phylogenetic analysis of the HA1 hemagglutinin gene segment based on nucleotide sequence. Tree was generated by general bootstrap analysis using 100 replicates and a heuristic search method with the PAUP 4.0b10 program. The outgroup used is DK/PA/69. Abbreviations used for identifying isolates: CK = chicken; DK = duck; JapQuail = Japanese quail; RT = ruddy turnstone; MotDuck = mottled duck; GWTeal = green-winged teal; RBGull = ring-billed gull.

chickens that became infected. One virus, CK/CA/6028/04, not only induced higher HI titers in chickens than in ducks, but more chickens seroconverted to that virus, indicating that it was more adapted to the chickens.

Most of the microscopic lesions observed in virus-infected chickens and ducks were confined to the respiratory tract (Tables 6, 7). All of the chickens examined (that were given the viruses studied) presented lesions in the nasal cavity, trachea, and lungs. In the nasal cavity, mild to moderate catarrhal or lymphocytic rhinitis and sinusitis, with mucocellular exudates containing sloughed epithelial cells, submucosal edema, and glandular hyperplasia were observed. The trachea presented mild to moderate degenerative changes of the overlying epithelium and mild lymphocytic infiltration in the submucosa, with mild edema. The lesions present in the lung consisted of mild congestion, mild interstitial inflammation with mixed mononuclear cells, mild to moderate catarrhal bronchitis, and mild proliferation of bronchiole-associated lymphoid tissues. Moderate, multifocal interstitial pneumonia was present in one of the chickens infected with CK/CA/431/00 and in two of the chickens infected with CK/CA/7211/04.

Minimal lymphocytic infiltration was present in the heart of two chickens infected with CK/CA/431/00 and CK/CA/6028/04, and one bird infected with CK/CA/7211/04 had moderate, diffuse lymphocytic pericarditis. All of the infected chickens examined had mild to moderate hyperplasia of the duodenum epithelium and mild proliferation of gut-associated lymphoid tissues. A mild to moderate increase in lymphocytic infiltrates in the periportal regions of the liver was observed in all infected chickens. Mild lymphocytic infiltration was also observed in the pancreas and gonads of some birds in all virus-inoculated groups. Also, minimal to mild lymphoid atrophy was observed in the bursa of Fabricius and thymus in some chickens in each group. Remaining organs lacked significant histopathologic lesions. No significant lesions were found in noninoculated chickens.

In ducks, similar to the chickens, most of the microscopic lesions were found in the upper respiratory tract (nasal cavity, trachea); however, different than seen in chickens, minimal or no lesions were observed in any other tissues including the enteric tract. Mild to moderate lymphocytic rhinitis and sinusitis, and mild to moderate tracheitis, was noted in all ducks inoculated with any of the viruses. Loss of trachea epithelium, with areas of squamous metaplasia, was commonly observed. Lesions in the lung were mild or nonexistent. Some ducks had mild congestion and minimal to mild mononuclear cell infiltration. One duck inoculated with CK/CA/6028/04 had moderate interstitial pneumonia. Negative control birds did not have any appreciable lesions.

Phylogenetically, the hemagglutinin gene of the H6N2 viruses from California all appeared to be part of a unique lineage with a common origin (Fig. 1). However, as previously described, comparison of the other gene segments showed multiple different lineages, providing evidence of reassortment with other influenza viruses (13). However, from a common origin of the H6 gene, genetic drift of the virus into three main groups can be observed. The viruses used in this study include representatives from all three groups. The earliest group of viruses, all isolated in 2000, is separated from the other isolates by a 2–4% sequence difference. The two other genetic groups are separated from each other by 3–7% nucleotide differences. The CK/CA/6028/04 isolate from a bird at a live bird market custom slaughter plant, and CK/CA/7211/04 from a bird in a nearby feed and pet supply store in Los Angeles, were closely related. The earliest isolate from California CK/CA/431/00 was more distant from the other California isolate. The CK/NY/14677-13/98 virus is genetically distinct from the California isolates, with approximately a 25% nucleotide sequence divergence. A previous study places CK/CA/139/01 and CK/CA/431/00 in sublineage II along with other H6N2 isolates from California and with one H6N1 isolate (A/pintail/Alberta/179/93) from Alberta (1).

In summary, these viruses can infect both chickens and ducks but, based on virus detection and histopathology, they are more adapted to infection and replication in chickens. No clear difference in pathology or virus shedding was observed between the H6N1 isolates from different years. Most infected chickens shed virus, but not all of the birds seroconverted. This is important because virus shedding in commercial chickens can lead to transmission of the virus among poultry, which could go unnoticed in the absence of clinical signs or detectable antibodies. In contrast, few of the ducks given the H6N1 viruses from California shed virus, but many of the birds seroconverted.

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